A novel siderophore system is essential for the growth of *Pseudomonas aeruginosa* in airway mucus

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A novel siderophore system is essential for the growth of *Pseudomonas aeruginosa* in airway mucus

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ABSTRACT

A novel siderophore system is essential for the growth of *Pseudomonas aeruginosa* in airway mucus

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Pseudomonas aeruginosa establishes airway infections in Cystic Fibrosis patients. Here, we investigate the molecular interactions between *P. aeruginosa* and airway mucus secretions (AMS) derived from the primary cultures of normal human tracheal epithelial (NHTE) cells. PAO1, a prototype strain of *P. aeruginosa*, was capable of proliferating during incubation with AMS, while all other tested bacterial species perished. A PAO1 mutant lacking *PA4834* gene became susceptible to AMS treatment. The Δ*PA4834* mutant was grown in AMS supplemented with 100 μM ferric iron, suggesting that the *PA4834* gene product is involved in iron metabolism. Consistently, intracellular iron content was decreased in the mutant, but not in PAO1 after the AMS treatment. Importantly, a PAO1 mutant unable to produce both pyoverdine and pyochelin remained viable, suggesting that these two major siderophore molecules are dispensable for maintaining

viability during incubation with AMS. The Δ*PA4834* mutant was regrown in AMS amended with 100 μM nicotianamine, a phytosiderophore whose production is predicted to be mediated by the *PA4836* gene. Infectivity of the Δ*PA4834* mutant was also significantly compromised *in vivo*. Together, our results identify a genetic element encoding a novel iron acquisition system that plays a previously undiscovered role in *P. aeruginosa* airway infection.

Key words: *PA4834* gene, siderophore system, airway mucus secretion

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Ⅰ. INTRUDUCTION

Pseudomonas aeruginosa is a highly adaptable Gram-negative bacterium that colonizes various environmental niches and causes major airway infections. Notably, 60-70% of patients with cystic fibrosis (CF) are infected by *P. aeruginosa* in the airway as the disease progresses to the age of 20.[1] As a major opportunistic pathogen, *P. aeruginosa* also infects patients suffering from ventilator-associated pneumonia[2] or burn wounds.[3] Previous studies demonstrated that thickened airway mucus caused by mutations in the cystic fibrosis transmembrane conductance regulator (*CFTR*) gene may be less effective in eliminating microbial invaders[4] due in part to decreased mucociliary clearance.[5] The concentrations of major ions in the airway mucus have been precisely determined and were not significantly different between normal and CF samples,[6] supporting the notion that isotonic volume changes in airway surface liquid accounts for defective mucociliary clearance followed by *P. aeruginosa* infection in the CF airway.[7] Furthermore, the abnormally altered CF airway was found to be anaerobic[8] and *P. aeruginosa* was found to form robust biofilms during

anaerobiosis.[9-11] However, these findings do not fully explain why *P. aeruginosa* has been exceptionally capable of establishing chronic airway infections.

Airway mucus contains various antibacterial components such as lysozyme,[12] lactoferrin[12] and IgA,[13] which suppress bacterial growth on the airway surface. Notably, elevated lysozyme activity and lactoferrin levels were observed in the bronchoalveolar lavage fluid (BALF) derived from CF patients.[14] In the same study, it was also shown that lysozyme and lactoferrin levels were increased in older CF patients.[14] These data suggest that the degree of *P. aeruginosa* infection may not correlate with the levels of these molecules in the CF airway and frequent *P. aeruginosa* infection is likely ascribed to its ability to effectively respond to host-specific hostile environments.

Iron is essential for bacterial survival and typical bacterial organisms require micromolar levels of iron for optimal growth.[15,16] However, the utilization of iron is limited by the host as most iron is bound to circulating proteins such as transferrin, lactoferrin, and ferritin *in vivo*.[17] To sequester iron for their own use, bacterial species possess a myriad of mechanisms that regulate the expression, secretion, and internalization of iron chelating compounds termed siderophores. Bacterial iron metabolism has been extensively studied using *P. aeruginosa* as a model organism. Pyochelin and pyoverdine are well-characterized siderophore molecules that *P. aeruginosa* produces under iron-limited conditions.[18] Siderophore-mediated processes also participate in virulence regulation of *P. aeruginosa*.[19,20] Intriguingly, pyoverdine-defective *P. aeruginosa* strains have been detected in CF sputa.[21,22] Moreover, a PAO1 mutant defective in both pyochelin and pyoverdine was found to colonize the lungs of immunocompromised mice, even though its virulence was attenuated.[23] These results indicate that additional iron-acquisition mechanisms may play a more important role during airway infection. In support of this notion, diverse iron acquisition pathways

have been reported in *P. aeruginosa*, including heme uptake systems,[24] TonB-dependent uptake systems,[25] and the FeOABC system.[26]

Airway mucus is a frontline defense of the innate immune response against invading bacterial pathogens; however, physiological changes induced in *P. aeruginosa* during interactions with airway mucus are not clearly understood at the molecular genetic level. In this study, we investigated various bacterial responses to airway mucus secretions (AMS) harvested from primary cultures of normal human tracheal epithelial (NHTE) cells. Unlike other bacterial species of clinical significance, *P. aeruginosa* exhibited resistance to treatment with AMS and was capable of replicating in its presence as well. We took a genome-wide approach to uncover a genetic determinant responsible for a previously uncharacterized iron uptake mechanism. This report provides novel insight into the interaction between *P. aeruginosa* and the host, especially at the early stages of airway infection. In addition, this work proposes a drug target, the inhibition of which may contribute to the efficient eradication of this important pathogen.

Ⅱ. MATERIALA AND METHODS

1. Experimental ethics

Experiments using human subjects and experimental animals were performed in strict accordance with guidelines provided by Yonsei University. Protocols were reviewed and approved by Institutional Review Board of Yonsei University College of Medicine. Permit numbers for primary culture of human tissues and mouse infection experiment were 2014-1842-001 and 2011-0173-2, respectively.

2. Bacterial strains, cell lines, and growth conditions

Bacterial strains and plasmids used in the current study are listed in the paragraph (Table 1). Luria-Bertani medium (LB; 10 g tryptone, 5 g yeast extract, and 10 g NaCl per liter) was used to grow bacterial precultures. For bacterial incubation with AMS, LB-grown bacterial cells were washed with PBS and diluted 10,000-fold into 100 μl of AMS placed in the wells of 96-well plates. Bacterial cells were then grown for 16 hrs in a humidified 37 °C incubator. Bacterial growth was assessed by measuring the growth index. Unless otherwise noted, the values for $\log_{10}(CFU_{\text{after 16 hrs in AMS or LB}}/CFU_{\text{after 16 hrs in PBS}})$ were calculated and plotted as the growth index. NuLi-1 (ATCC® CRL-4011™) and CuFi-1 (ATCC® CRL-4013™) cell lines were purchased from ATCC (Rockville, MA). Cell line maintenance and cultures were conducted following standard tissue culture techniques. Transposon insertion mutants of *P. aeruginosa* were purchased from a *P. aeruginosa* transposon mutant library (http://www.genome.washington.edu/UWGC/pseudomonas). The targeted gene mutations were introduced by allele replacement as previously described[11].

Table 1. Bacterial strains and plasmids used in this study

Strains or plasmids	Relevant characteristics	Ref. or source
P. aeruginosa strains		
PAO1	Wild-type PAO1, ATCC	Lab collection
Δ*PA4834*	PA01, *PA4834* deleted	This study
Δ*pvd*	PAO1, *PA2396* and *PA2397* deleted	This study
Δ*pch*	PAO1, *PA4224* and *PA4225* deleted	This study
Δ*pvd*Δ*pch*	PAO1, *PA2396, PA2397, PA4224* and *PA4225* deleted	This study
Δ*PA4834*Δ*pvd*	PA01, *PA4834, PA2396* and *PA2397* deleted	This study
Δ*PA4834*Δ*pch*	PA01, *PA4834, PA4224* and *PA4225* deleted	This study
Δ*PA4834*Δ*pvd*Δ*pch*	PA01, *PA4834, PA2396, PA2397, PA4224* and *PA4225* deleted	This study
Δ*PA4834*::Tn	*PW9128, PA4834*:: ISlacZ/hah, UW strain	[27]
Δ*PA4834*/pJN105	Δ*PA4834*, empty vector control	This study
Δ*PA4834*/pJN105::*PA4834*	Δ*PA4834* complementation strain	This study
E. coli strains		
SM10/λpir	Km^r *thi-1 thr leu tonA lacY supE recA*::RP4–2-Tc::Mu *pir*$^+$, for conjugal transfer	[28]
Other bacterial species		
Staphylococcus aureus		Lab collection
Bacillus cereus		Lab collection
Escherichia coli	DH5α	Lab collection
Vibrio cholerae	N16961	Lab collection
Salmonella enterica serovar Typhimurium	LT2	Lab collection
Listeria monocytogenes		Lab collection
Plasmids		
pCVD442	*sacB* suicide vector from plasmid pUM24	[28]
pJN105	pJN105 *araC*-PBAD cassette cloned in pBBR1MCS-5, Gm^r	[29]
pJN105::*PA4834*	pJN105 inducible vector with fragment of *PA4834*	This study

3. Preparation of airway mucus secretions (AMS)

AMS was collected from primary cultures of normal human tracheal epithelial (NHTE) cells following procedures described previously.[30] In brief, a piece of human trachea was harvested from patients without airway disease during tracheotomy. Second passage NHTE cells (10^5 cells / culture) were seeded in 0.5 ml of culture medium onto 24.5 mm, 0.45 mm pore size Transwell® clear culture inserts (Costar Co., Cambridge, MA). Cultured cells were grown submerged, and the culture medium was changed on the first day and every other day thereafter. After cultures reached confluence, media on the luminal side was removed to create an air–liquid interface (ALI) to facilitate differentiation into ciliated columnar epithelial cells with polarity. To harvest AMS, we washed the apical surface of the NHTE cells with 1 ml PBS. All patients provided informed consent, and the Institutional Review Board of Yonsei University College of Medicine approved this study (2014-1842-001).

4. Complementation of the Δ*PA4834* mutant

A 0.86-kb NdeI-HindIII fragment containing the entire *PA4834* gene was amplified from the PAO1 genome and ligated into NdeI/HindIII-treated pET21b. To complement the Δ*PA4834* mutant, the arabinose-inducible expression vector pJN105[29] was used. PCR was used to amplify the *PA4834* gene along with the ribosome binding site (RBS) that originated from pET21b. The EcoRI/XbaI-digested PCR product was ligated into EcoRI/XbaI-digested pJN105. The resulting plasmid and the control empty plasmid (i.e., pJN105) were transferred into the Δ*PA4834* mutant by electroporation.

5. Microarray analysis

Genome-wide transcriptome analysis was performed using GeneChip *P. aeruginosa* genome arrays (Affymetrix, Santa Clara, CA). PBS-washed PAO1 cells

precultured in LB overnight were incubated for 6 hrs in PBS or in AMS. RNA extraction, labeling, hybridization, and data analysis were performed as described elsewhere.[11]

6. Antibiotic sensitivity assay

Commercially purchased filter discs (BBL Sensi-Disc susceptibility test discs; Becton, Dickinson and Company, Franklin Lakes, NJ) were used. After overnight incubation of PAO1, and Δ*PA4834* mutant cells on LB agar plates containing various bacteria the zones of inhibition were measured.

7. Quantitative real time PCR

PA4834, *PA4835*, *PA4836*, and *PA4837* gene transcript levels were determined by qRT-PCR, following previously described procedures[11]. Prior to RNA extraction, PAO1 cells were grown for 6 hrs in 1 mL of LB or AMS with shaking at 37°C. Transcript levels of the *rpoD* gene were also measured and used for normalizing *PA4834*, *PA4835*, *PA4836*, and *PA4837* gene transcription. The primers used for qRT-PCR are listed in Table 2.

Table 2. Primers used in this study

Gene name	Direction	Primer sequence (5'-3')[a]	Restriction sites
Mutant construction			
PA4834 left	Forward	ATATAGAGCTCGCCGAGCGCCAGGAATACCTG	SacI
PA4834 left	Reverse	TAAATGGATCCCAGCAGGGTACCGATGGCGC	BamHI
PA4834 right	Forward	CCCTTGGATCCGGCATCGTCTTCAACGCCGG	BamHI
PA4834 right	Reverse	AAGGTGAGCTCACGCCTTTCTGCCTGGTCACG	SacI

pvd left	Forward	ACACGGAGCTCCCCTTCCCGTTCGACGAAGGTA	SacI
pvd left	Reverse	CTTCCGGATCCTTCACCCGACGACACCATCCTC	BamHI
pvd right	Forward	AGAGCGGATCCAATGCCTGGCTCGAAGAGCGAC	BamHI
pvd right	Reverse	CGATTGAGCTCACTCCTGGAGTGCGCTGCCC	SacI
pch left	Forward	ACACGGAGCTCAATGGCAAGGTCGATCGTCGC	SacI
Pch left	Reverse	CTTCCGGATCCCAGGTGTTCCAGCAGGGCCTC	BamHI
pch right	Forward	AGAGCGGATCCAACCAGCGCAGCCTGGAGGT	BamHI
pch right	Reverse	CGATTGAGCTCAGCGTGCGCGATCAGTTGGT	SacI
complementation			
PA4834 complementation (cloning to pET21b)	Forward	CTTACCATATGGTGCTCGACCTGCTGAAGAGC	NdeI
	Reverse	AAATTAAGCTTGCCCTTCTTCGTCCGCGACCAG	HindIII
PA4834 complementation (cloning to pJN105)	Forward	ACTCTGAATTCAATAATTTTGTTTAACTTTAAGAAGGAG	EcoRI
	Reverse	ATTGGTCTAGATCAGTGGTGGTGGTGGTG	XbaI
Quantitative RT-PCR			
PA4834	Forward	GACCTGCTGAAGAGCGGGGT	
	Reverse	GCTTCCTTGACCAGGGCGTT	
PA4835	Forward	TACGTGCACAGTCCGTTCTT	
	Reverse	GATAGGTCCCTCCGGGTAGA	
PA4836	Forward	CTGTTCAACTATCCGGTGGAG	
	Reverse	TTCTCCAGGATCAGGGTGTC	
PA4837	Forward	ACCTTGACAGCGCTCGTATC	
	Reverse	AAGCCACGGACGTTGTACTC	
rpoD (PA0576)	Forward	AAGGCCCTGAAGAAGCACGG	
	Reverse	GATCGGCATGAACAGCTCGG	

8. Scanning electron microscope

Primary cultured NHTE cells grown on permeable membrane (Costar Co., Cambridge, MA) were inoculated with overnight cultured PAO1, SA or Δ*PA4834* mutant (2×10^4 CFU in 300 μl PBS). After 14 hrs incubation, the NHTE cells were fixed, dried and coated with platinum using a sputter coater (E1030; Hitachi).Coated specimens were then mounted on a stub holder and viewed using a cold-field emission scanning electron microscope (S-4300, Hitachi) operated at 15 kV.

9. Intracellular iron concentration

Inductively coupled plasma mass spectrometry (ICP-MS) was performed to determine the intracellular concentration of total iron. Bacterial pellets grown in LB or AMS were submitted to the Korea Basic Science Institute (Seoul, Korea), where subsequent procedures were performed. The cell-permeable green fluorescent heavy metal indicator, PhenGreen™ SK, diacetate (Molecular Probes, Inc., Eugene, OR) was used to fluorescently stain bacterial cells. Aliquots of bacterial suspensions were stained with 20 μM PhenGreen™ SK, diacetate for 30 min and fluorescent images were acquired using a fluorescent microscope (Zeiss LSM 700; Carl Zeiss Inc., Germany).

10. Iron sequestration assay

Bacterial strains were grown in LB and cell-free culture supernatants were subject to the iron sequestration assay using a colorimetric iron assay kit according to the manufacturer's instructions (SideroTech Kit. Emergen Bio Inc., Ireland). The assay is based on the color change that occurs as result of ferric iron transfer from the reagent complex to siderophore present in culture supernatants.

11. Mouse infection

Methods for animal infection were approved by the Yonsei University College of Medicine Committee on the Ethics of Animal Experiments (permit number 2011-0173-2). Effects of the *PA4834* gene deletion on *P. aeruginosa in vivo* infectivity was monitored using two different mouse infection models, the thermally-injured mouse infection model[31] and the acute airway infection[32] model. For thermal injury, adult male C57BL/6 mice were anaesthetized and their backs were shaved. Thermal injury was induced by exposing the shaved skin (15% of their body surface) to an aluminum plate (16 mm diameter) heated to 99°C for 8 sec. Immediately after, sterilized gauze was fixed on the injured skin and the mice were then challenged by spraying 0.2 ml of 2×10^8 *P. aeruginosa* CFU or 0.2 ml PBS (as the control) on the gauze. Acute airway infection was performed following the procedures described previously.[32] For both infection experiments, affected tissues were recovered and stained with hematoxylin-eosin for histological analysis.[32]

12. Statistical analysis.

Data are expressed as the mean ± standard deviation (SD). An unpaired Student's *t*-test was used to analyze the data. A p value of < 0.05 was considered statistically significant. All the experiments were repeated for reproducibility.

Ⅲ. RESULTS

1. *P. aeruginosa* exhibits exceptional resistance in response to incubation with airway mucus secretions (AMS)

Airway mucus contains a variety of antimicrobial agents[33] serving as a frontline immune defense against invading microorganisms. We first examined how AMS exerts its effect on various bacterial species including human pathogens by comparing the number of viable cells before and after incubation with AMS. Bacterial growth was monitored by measuring the growth index as described in the Methods section. After 16 hrs incubation with AMS, all of the tested bacterial species, except for *P. aeruginosa*, lost their viability. *S. aureus* was the most susceptible to the treatment with AMS. In contrast, the growth index of PAO1, the prototype *P. aeruginosa* strain, was increased after the same treatment (Fig. 1A).

Next, we measured bacterial growth in the presence of secretory products recovered from the culture of two different human airway epithelial cell lines, NuLi-1 (normal airway epithelial cell line) and CuFi-1 (cystic fibrosis airway epithelial cell line). The antibacterial activities of these secretions were found be weaker than the AMS prepared from the primary cultures of NHTE cells, and both secretions only partially inhibited *S. aureus* growth (Fig. 1B). No significant difference was observed between the two secretions in inhibiting the growth of *S. aureus*. Likewise, PAO1 growth was not affected at all by incubation with each of the two secretions, further suggesting that PAO1 can develop full resistance to the secretions of NuLi-1 or CuFi-1 cells (Fig. 1B). Together, these results demonstrate that *P. aeruginosa* both resists the antimicrobial activity of AMS and also propagates during incubation with AMS.

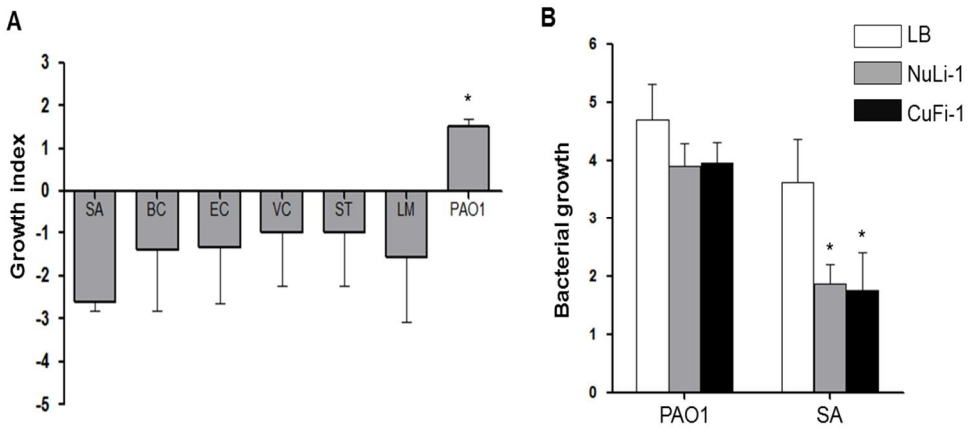

Figure 1. Robust ability of *P. aeruginosa* to grow in airway mucus. (A) Seven different bacterial strains (SA, *Staphylococcus aureus*; BC, *Bacillus cereus*; EC, *Escherichia coli*; VC, *Vibrio cholerae*; ST, *Salmonella enterica* serovar Typhimurium; LM, *Listeria monocytogenes*; and PAO1, *P. aeruginosa*) were incubated with AMS prepared from primary cultures of normal human tracheal epithelial cells for 16 hrs. Changes in bacterial cell viability were monitored by calculating the growth index as described in the Materials and Methods. Four independent experiments were performed, and the mean values ± SD (error bars) are displayed in each bar. (B) *P. aeruginosa* strain PAO1 (PA) and *S. aureus* (SA) cells were incubated with secretions collected from cultured NuLi-1 (gray bars) and CuFi-1 (black bars) cells. Bacterial growth in LB media was also measured (white bars) and compared with other growth. Four independent experiments were performed, and the mean values ± SD (error bars) are displayed in each bar. *$p < 0.05$ vs. SA growth in LB media.

2. Genome-wide expression analysis followed by mutant screening identified a PAO1 mutant susceptible to AMS treatment

Our results show that PAO1 is exclusively resistant to the antimicrobial action of AMS, which *P. aeruginosa* cells likely encounter during the early stages of the airway infection. To offer clues into the molecular basis of this unique resistance, we performed a microarray analysis. Table 3 shows a list of the top 35 genes that were most upregulated during incubation with AMS vs. PBS. Exposure to AMS influenced bacterial transcription dramatically, as evidenced by the extent to which expression was changed (~7- to ~56-fold increase). A substantial number of genes (16 genes) were determined to encode hypothetical proteins. Of note, four genes (*pchB*, *pchG*, *pchE*, and *pchF*) involved in the biosynthesis of pyochelin, a siderophore molecule for iron acquisition,[34] were included in the list. Furthermore, expression of the *pvdS* gene encoding a sigma factor that activates the production of pyoverdine, another iron chelator,[35] was also highly induced in PAO1 cells grown in the presence of AMS. Transcript levels of two genes (*PA4834* and *PA4837*) constituting an operon were also highly induced in response to AMS treatment (Table 3).

Table 3. List of genes that are most upregulated during growth with AMS

PA number	Gene	Fold change [a]	Target Description
PA3446		56.63	conserved hypothetical protein
PA3600		49.03	conserved hypothetical protein /Translation, post-translational modification
PA0284		36.28	hypothetical protein
PA4063		35.15	hypothetical protein
PA3601		24.27	conserved hypothetical protein /Translation, post-translational modification
PA2359		21.93	probable transcriptional regulator
PA3450		21.63	probable antioxidant protein /adaptation, protection
PA4570		19.36	hypothetical protein
PA4471		18.45	hypothetical protein
PA2161		16.99	hypothetical protein
PA0802		16.82	hypothetical protein /Membrane proteins
PA5536	*dksA2*	15.69	DnaK suppressor protein
PA2062		15.52	probable pyridoxal-phosphate dependent enzyme /Putative enzymes
PA1911	*femR*	13.79	sigma factor regulator
PA2033		11.83	hypothetical protein
PA1905	*phzG2*	11.29	probable pyridoxamine 5'-phosphate oxidase /Secreted Factors
PA4230	*pchB*	10.98	salicylate biosynthesis protein PchB /Transport of small molecules; Secreted Factors
PA1373	*fabF2*	10.86	3-oxoacyl-acyl carrier protein synthase II /Fatty acid and phospholipid metabolism
PA4103		10.61	hypothetical protein
PA4224	*pchG*	10.61	pyochelin biosynthetic protein PchG
PA2570	*lecA*	10.15	PA-I galactophilic lectin /Adaptation, protection; Motility & Attachment Cell wall
PA2786		9.98	hypothetical protein
PA1300		9.66	probable sigma-70 factor, ECF subfamily /Transcriptional regulators
PA3381		9.55	probable transcriptional regulator /Transcriptional regulators
PA0993	*cupC2*	9.36	chaperone CupC2/probable pili assembly chaperone /Motility & Attachment
PA4033	*mucE*	9.01	hypothetical protein

PA4837		8.95	probable outer membrane protein precursor/Membrane proteins
PA4516		8.93	hypothetical protein
PA4834		8.33	hypothetical protein /Membrane proteins
PA4226	*pchE*	8.27	dihydroaeruginoic acid synthetase /Transport of small molecules; Secreted Factors
PA0790		8.05	hypothetical protein /Membrane proteins
PA3126	*ibpA*	7.92	heat-shock protein LbpA /Chaperones & heat shock proteins
PA1312		7.84	probable transcriptional regulator /Transcriptional regulators
PA2426	*pvdS*	7.76	sigma factor PvdS /Transcriptional regulators
PA4225	*pchF*	7.23	pyochelin synthetase /Transport of small molecules; Secreted Factors

[a]Fold change was based on expression level in PAO1 incubated in PBS for the same period of time.

Next, we assessed the effect of disruption of each gene on the susceptibility of PAO1 to AMS treatment. Transposon (Tn) insertion mutants were grown in AMS and the growth index of each mutant was calculated (Table 4). All of the mutants grew fine in LB media (data not shown). While the 31 tested mutants exhibited varying degrees of growth, most of them showed positive growth index values (Table 4). Of importance is that mutations in either *PA4063* or *PA4834* resulted in growth inhibition in our initial screen (growth index < 0). Subsequent experiments indicated that the *PA4834* Tn mutant was consistently susceptible to treatment with AMS. To further validate this result, we constructed an in-frame deletion mutant of *PA4834* and the mutant also lost its viability when treated with AMS (growth index of approximately -1.0), although the degree of viability loss was not as significant as what was observed in the *S. aureus* strain (Fig. 2A). Importantly, restored ability to grow in AMS was observed when the mutant was complemented with a plasmid-born wild-type copy of the *PA4834* gene (Fig. 2A). For the investigation of bacterial colonization in NHTE cells, primary NHTE cells were differentiated at an air–liquid interface (ALI) for 8 days. PAO1, SA and Δ*PA4834* mutant were inoculated into the apical region of the

NHTE cells and incubated for 14 hrs. Fig. 2B represents scanning electron microscope (SEM) images of infected NHTE cells with seeded bacterial cells. PAO1 invaded the NHTE cells and expanded (Fig. 2B). However SA and Δ*PA4834* mutant fewer in numbers and did not affect the NHTE cells (Fig. 2B). *PA4834* is a component of four-gene operon (*PA4834-PA4837*). Each gene of the operon was evaluated for its expression. Quantitative RT-PCR analysis showed that PAO1 have 11, 10, 9, 19-fold increased levels of *PA4834*, *PA4835*, *PA4836*, and *PA4837* gene transcripts, respectively, when grown in AMS compared to LB. This result suggests that the *PA4834-PA4837* may be operon, and *PA4834* plays an important role in the interaction with AMS (Fig. 2C).

Table 4. Effects of the disruption of upregulated genes on sensitivity to AMS treatment

Strains	Growth index	Strains	Growth index
PAO1	2.46 (±0.61)	PA1373::Tn	1.48
PA3446::Tn	2.23	PA4103::Tn	1.75
PA3600::Tn	1.71	PA4224::Tn	1.16
PA0284::Tn	1.77	PA2570::Tn	1.34
PA4063::Tn	**-0.06**	PA2786::Tn	not tested
PA3601::Tn	1.92	PA1300::Tn	1.45
PA2359::Tn	1.54	PA3381::Tn	1.03
PA3450::Tn	not tested	PA0993::Tn	1.45
PA4570::Tn	1.58	PA4033::Tn	2.20
PA4471::Tn	1.18	PA4837::Tn	1.93
PA2161::Tn	1.54	PA4516::Tn	1.90
PA0802::Tn	1.85	**PA4834::Tn**	**-0.18**
PA5536::Tn	2.31	PA4226::Tn	2.94
PA2062::Tn	1.54	PA0790::Tn	2.34

PA1911::Tn	1.32	PA3126::Tn	3.56
PA2033::Tn	not tested	PA1312::Tn	3.79
PA1905::Tn	1.53	PA2426::Tn	3.50
PA4230::Tn	not tested	PA4225::Tn	4.00

PA4834 is a component of a four-gene operon (*PA4834-PA4837*) and is predicted to encode a membrane-associated protein (www.pseudomonas.com). The *PA4836* and *PA4837* genes encode proteins homologous to nicotianamine synthase and a TonB-dependent siderophore receptor, respectively (Table 5). Table 5 shows a list of bacterial species that were found to possess genes homologous to each component of the operon. *P. aeruginosa* strains contain all four genes, each with >99% sequence identity to the corresponding gene in PAO1. Sequence identities between cognate genes in PAO1 vs. PA7 were somewhat lower (91~96 %) at the nucleotide level (Table 5). *Serratia* sp. FS14 was found to harbor two genes similar to *PA4834* and *PA4835*, with higher identity to the *PA4834* gene. It is of interest that these two genes also constitute a likely six-gene operon, predicted to be involved in iron metabolism (data not shown). Other genomes on the list contain only one gene homologous to a corresponding gene of the *PA4834-PA4837* operon (Table 5). Of note, the bacterial species tested in Fig. 1A were determined not to possess genetic elements similar to any part of the *PA4834-PA4837* operon. Together, these results suggest that the *PA4834-PA4837* operon is exclusively found in *P. aeruginosa*, and not in other bacterial species with sequenced genomes.

Table 5. BLASTN search of *PA4834*, *PA4835*, *PA4836* and *PA4837* gene sequences

Genes	**PA4834**	**PA4835**	**PA4836**	**PA4837**
Predicted function	EamA-like transporter family protein	Conserved uncharacterized	Nicotianamine synthase	TonB-dependent siderophore receptor
Bacterial species that contain genes homologous to *PA4834*, *PA4835*, *PA4836* or *PA4837*				
P. aeruginosa PA01	855 / 855 (100%)	1302 / 1302 (100%)	792 / 792 (100%)	2127 / 2127 (100%)
P. aeruginosa DK2	855 / 855 (100%)	1296 / 1302 (99%)	787 / 792 (99%)	2123 / 2127 (99%)
P. aeruginosa LESB58	853 / 855 (99%)	1295 / 1302 (99%)	784 / 792 (99%)	2114 / 212 7 (99%)
P. aeruginosa M18	853 / 855 (99%)	1296 / 1302 (99%)	786 / 792 (99%)	2120 / 2127 (99%)
P. aeruginosa RP73	853 / 855 (99%)	1297 / 1302 (99%)	786 / 792 (99%)	2118 / 2127 (99%)
P. aeruginosa PA96	854 / 855 (99%)	1299 / 1302 (99%)	785 / 792 (99%)	2115 / 2127 (99%)
P. aeruginosa UCBPP-PA14	846 / 854 (99%)	1286 / 1302 (99%)	783 / 792 (99%)	2108 / 2127 (99%)
P. aeruginosa PA7	788 / 855 (92%)	1188 / 1302 (91%)	757 / 792 (96%)	2001 / 2127 (94%)
P. protegens Cab57				1436 / 2007 (72%)
P. stutzeri A1501				1465 / 2062 (71%)
P. mendocina ymp				1478 / 2094 (71%)
P. mandelii JR-1				1432 / 2017 (71%)
Burkholderia sp. 383	577 / 828 (70%)			
Serratia sp. FS14	530 / 782 (68%)	255 / 373 (68%)		
Serratia marcescens WW4	514 / 759 (68%)			
Arsenophonus nasoniae	487 / 726 (67%)			
Vibrio nigripulchritudo str. SFn1	250 / 385 (65%)			
Actinosynnema mirum DSM 43827		207 / 285 (73%)		
Bordetella bronchiseptica 253			38 / 43 (88%)	
Bordetella bronchiseptica MO149			38 / 43 (88%)	

Azotobacter vinelandii CA6	1480 / 2023 (73%)
Azotobacter vinelandii CA	1480 / 2023 (73%)

The Δ*PA4834* mutant grew completely fine in LB unlike AMS. And in our result of antibiotic sensitivity assay, the mutant exhibited an equal sensitivity to antibiotics with its parental strain PAO1 (Table 6). Δ*PA4834* mutant had no change in fitness or stability as a consequence of gene manipulation. The responses of PA4834 to AMS are due to the functional defect in the PA4834 gene.

Table 6. Antibiotic susceptibility test

Antibiotics	Inhibition zone (mm)	
	PA01	Δ*PA4834*
Amikacin	24	26
Ceftazidime	27	29
Ciprofloxacin	34	33
Gentamicin	19	21
Tobramycin	26	26
Cefoperazone-sulbactam	27	27
Piperacillin	28	28
Imipenem	24	26
Piperacillin-tazobactam	29	30
Aztreonam	26	27
Meropenem	31	31
Cefepime	30	31
Colistin	13	13

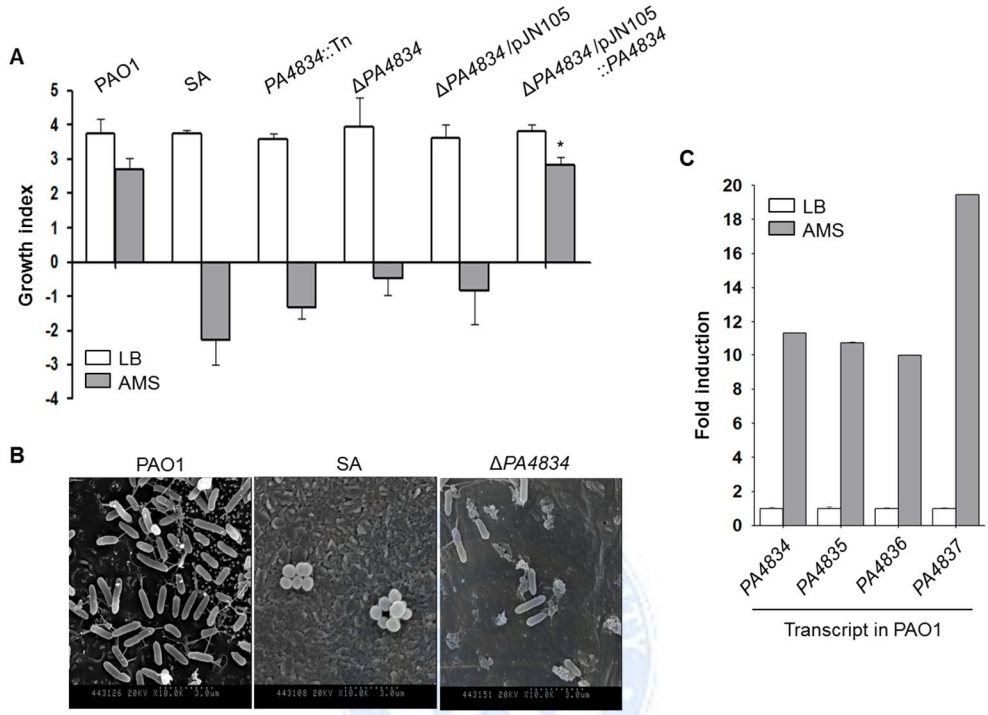

Figure 2. A PAO1 mutant devoid of the *PA4834* gene lost its ability to grow in AMS. (A) Bacterial strains indicated at the top were grown in LB (white bars) and in AMS (gray bars), and the growth index for each strain was measured. Experimental conditions were identical to those described in Fig. 1A. Five independent experiments were performed, and mean values ± SD (error bars) are displayed in each bar. SA, *S. aureus*. *$p < 0.05$ vs. growth indices of *PA4834*::Tn, Δ*PA4834*, and Δ*PA4834*/pJN105. (B) Scanning electron microscope of primary cultured NHTE cells infected with PAO1, SA and Δ*PA4834* mutant. Images are original magnification; 10,000× (C) Quantitative real-time PCR was performed to assess *PA4834*, *PA4835*, *PA4836*, and *PA4837* gene transcript levels. PAO1 cells were grown in LB or AMS before RNA extraction. Transcript levels of the *PA4834*, *PA4835*, *PA4836*, and *PA4837* genes in LB-grown PAO1 were normalized to 1.0, respectively. Three independent experiments were performed, and mean values ± SD are displayed in each bar.

3. The *PA4834* gene product is essential for iron acquisition during the interaction with AMS

Our microarray analysis indicated that genes involved in iron metabolism were highly transcribed in response to AMS treatment. Moreover, *PA2033*, annotated as a hypothetical gene, is predicted to encode a protein homologous to a siderophore-interacting protein (www.pseudomonas.com). All of this information led us to hypothesize that PAO1 likely activates its response to acquire iron during treatment with AMS, and *PA4834* gene deficiency may result in the failure of such a process. In line with this notion, AMS was reported to contain large amounts of lactoferrin[12,36] and transferrin,[37] which can sequester free iron to restrict bacterial growth.

When the Δ*PA4834* mutant was grown in AMS supplemented with 100 μM ferric iron, its viability was completely recovered (Fig. 3A). The growth index of wild-type PAO1 was comparable in the presence or absence of added $FeCl_3$. We then compared the relative sensitivity of PAO1 and the mutant under iron-depleted conditions. PAO1 showed better resistance to the presence of increasing concentrations of 2,2'-bipyridyl, an iron chelator. At a 1.6 mM concentration, PAO1 grew significantly better than the Δ*PA4834* mutant (Fig. 3B). ICP-MS analysis is a useful technique to measure total iron concentration in bacterial cells.[38] Intracellular iron concentration of the mutant was only slightly lower than PAO1, when strains were grown in LB (Fig. 3C). However, a dramatic difference was induced when the strains were grown in AMS. While a significantly increased level of intracellular iron was detected in AMS-grown PAO1, iron concentration in the Δ*PA4834* mutant was considerably decreased under the same growth condition (Fig. 3C). This result suggests that PAO1 cells, but not the mutant cells, are capable of activating cellular machineries to uptake iron. Likewise, when bacterial cells were stained with PhenGreen™ SK, diacetate, a green-fluorescent heavy metal indicator, a sharp increase in green fluorescent signal was detected in PAO1 grown in AMS vs. LB (Fig. 3D). Consistent with the ICP-MS

results, such an increase was not observed in the Δ*PA4834* mutant, further demonstrating that the possession of intact *PA4834* gene is crucial for adjusting bacterial physiological status to an adverse environment, where iron limitation is involved. To compare the relative siderophore activities of bacterial species, iron sequestration assay was performed using SideroTech Kit (Fig. 3E). The result demonstrated that (i) PAO1 produced by far the largest amount of siderophore and (ii) all other species found to be susceptible to the AMS treatment (Fig. 1) produced significantly lower levels of siderophore. This new result further suggests that the resistant growth of *P. aeruginosa* in AMS is strongly related with its robust iron sequestration activity. Importantly, iron sequestration activity was ~2-fold decreased in the Δ*PA4834* mutant, which was completely restored by *PA4834* gene complementation.

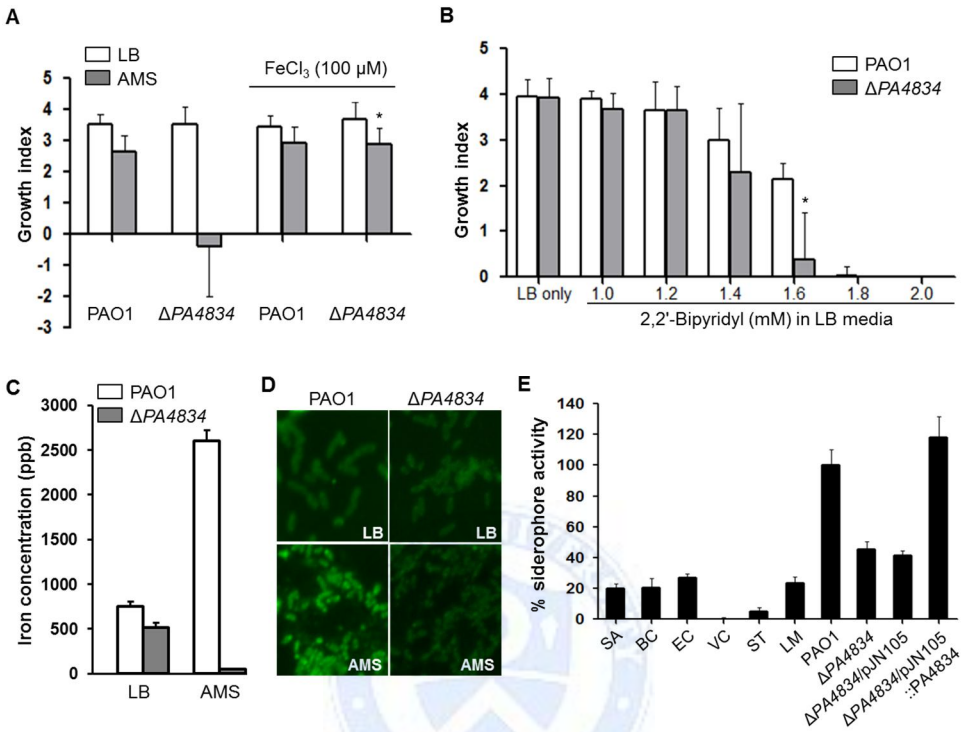

Figure 3. The *PA4834* gene product plays a role in iron acquisition. (A) Effect of ferric iron on growth of the Δ*PA4834* mutant in AMS. Growth indices of PAO1 and the Δ*PA4834* mutant after growth in LB (white bars) or in AMS (grays bars) in the absence or presence of 100 μM $FeCl_3$. Experimental conditions were identical to those described in Fig. 1A. Six independent experiments were performed, and mean values ± SD (error bars) are displayed in each bar. *$p < 0.05$ vs. growth of the Δ*PA4834* mutant in AMS without extraneously added $FeCl_3$. (B) Effect of an iron chelator on the growth of PAO1 and the Δ*PA4834* mutant. Growth indices of PAO1 (white bars) and the Δ*PA4834* mutant (gray bars) were calculated after growth in LB supplemented with increasing concentrations of 2, 2'-bipyridyl. Bacterial cells were grown for 12 hrs. Four independent experiments were performed, and mean values ± SD (error bars) are displayed in each bar. *$p < 0.05$ vs. growth of PAO1 in the same media. (C) Intracellular iron concentration of PAO1 (white bars) and the Δ*PA4834* mutant (gray bars) after growth in LB or in AMS. Inductively coupled plasma mass spectrometry (ICP-MS) was used for the measurement and iron concentrations are displayed in parts per billion (ppb). (D) Fluorescent microscope images of PAO1 and the Δ*PA4834* mutant. After growth in LB or AMS, bacterial suspensions were stained

with PhenGreen™ SK, diacetate, a green fluorescent heavy metal indicator. Experiments were repeated three times and representative images, processed at the same magnification, are shown. (E) Bacterial strains indicated at the bottom were grown in LB for 16 hrs and cell-free culture supernatants were subject to the iron sequestration assay. The iron sequestration activities of indicated species were normalized with that of PAO1.

4. Pyochelin and pyoverdine are dispensable for maintaining viability during incubation with AMS

Our results demonstrate that expression of *PA4834* is substantially stimulated upon exposure to AMS, which likely encodes a protein critical for iron acquisition. In *P. aeruginosa*, two major siderophore molecules, pyochelin and pyoverdine, have been extensively studied to determine their roles in iron metabolism.[15,39,40] As was shown in our microarray results, expression of genes involved in the production of these two molecules was also upregulated in response to treatment with AMS. We therefore sought to examine the relative importance of each system in terms of responsiveness to AMS. To address this issue, we constructed a series of mutants and calculated the growth index of each mutant. *P. aeruginosa* single mutants deficient in the production of either pyochelin or pyoverdine were completely viable in response to AMS treatment (Fig. 4A). Importantly, a Δ*pvd*Δ*pch* double mutant defective in both pyochelin and pyoverdine also remained viable in response to the same treatment. Of note, the growth indices of the Δ*pvd*, Δ*pch*, or Δ*pvd*Δ*pch* mutants were almost identical to that of PAO1, suggesting that the ability to produce these two siderophores is not required for the protective response to AMS. In contrast, when the *PA4834* gene was additionally disrupted, all the mutants (Δ*pch*Δ*PA4834*, Δ*pvd*Δ*PA4834* and Δ*pvd*Δ*pch*Δ*PA4834*) were susceptible to the antimicrobial action of AMS (Fig. 4A).

We next examined whether the susceptible Δ*PA4834* mutant could be rescued from AMS treatment by supplementation with bacterial culture supernatants. As shown in Fig. 4B, when the Δ*PA4834* mutant was grown in AMS amended with cell-free culture supernatants from the Δ*pvd*Δ*pch* mutant, its growth index was significantly increased. Culture supernatant of the triple mutant, however, failed to rescue the Δ*PA4834* mutant (Fig. 4B) and the growth index was quite similar to that of the mutant grown in AMS. This result further demonstrates that secretory

molecule(s), the production of which is dependent on the presence of the *PA4834* gene product, is critically necessary for the survival and proliferation of *P. aeruginosa* in AMS.

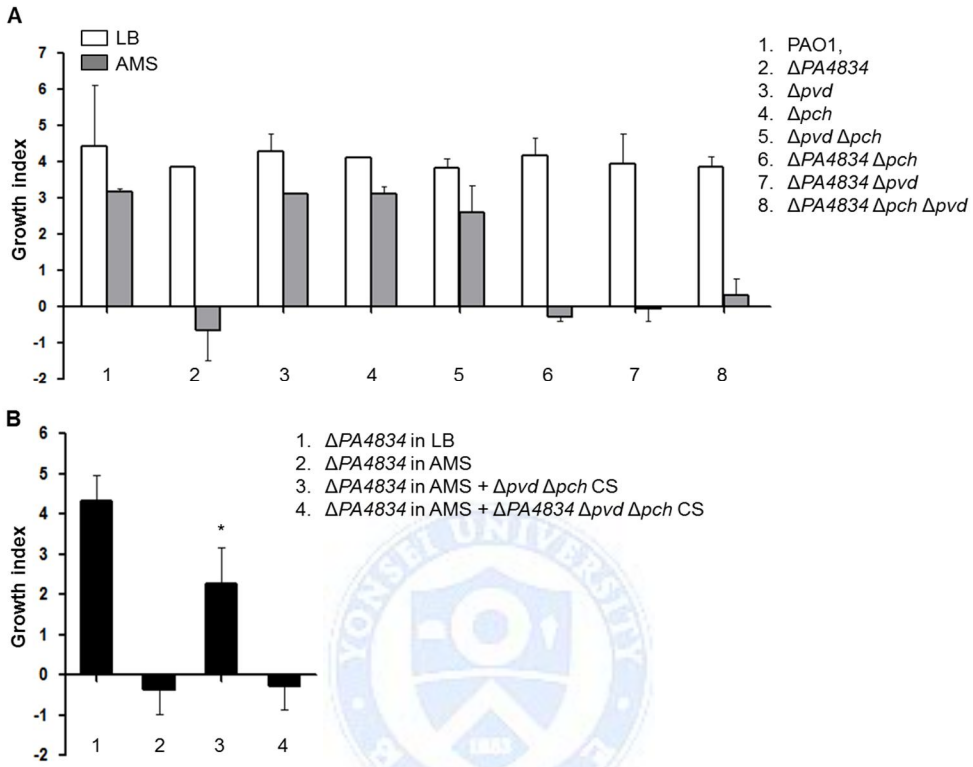

Figure 4. Growth of PAO1 pyochelin and pyoverdine mutants in AMS. (A) Bacterial strains (indicated by numbers) were grown in LB (white bars) or AMS (gray bars) for 16 hrs and the growth index of each strain was measured. Experimental conditions were identical to those described in Fig. 1A. Three independent experiments were performed, and mean values ± SD (error bars) are displayed in each bar. (B) Effect of bacterial culture supernatant (CS) on Δ*PA4834* mutant growth in AMS. The Δ*PA4834* mutant was grown in LB (white bar) and AMS supplemented with the CS from the Δ*pvd*Δ*pch* double or *ΔPA4834ΔpvdΔpch* triple mutant (gray bars). CSs were obtained from bacterial cultures grown in LB and diluted 10-fold in AMS. *$p < 0.05$ vs. growth of the Δ*PA4834* mutant in AMS or AMS amended with the CS from the *ΔPA4834 ΔpvdΔpch* mutant.

5. Supplementation of nicotianamine (NA) restores ability of the Δ*PA4834* mutant to grow in AMS

The *PA4836* gene encodes a putative NA synthase. NA is a compound with high-affinity for iron and it has been extensively studied in plants.[41,42] Furthermore, the *PA4837* gene product likely acts as a siderophore receptor suggesting the involvement of this genetic element in iron acquisition. This information led us to postulate that *P. aeruginosa* may also produce NA to sustain its growth in AMS. To address this issue, we examined whether NA supplementation could rescue growth of the Δ*PA4834* mutant in AMS. Similar to previous results, PAO1 was capable of growing in AMS regardless of the presence of 100 μM NA (Fig. 5). Importantly, growth of the Δ*PA4834* mutant was completely restored by the presence of 100 μM NA (Fig. 5).

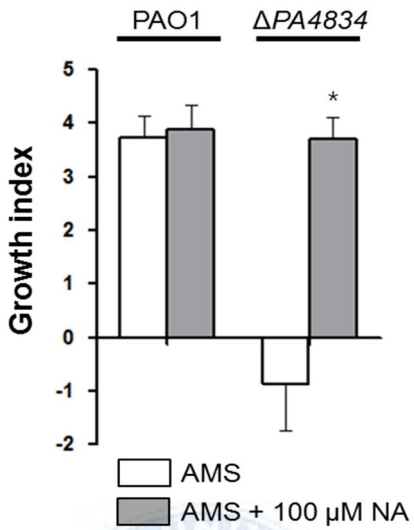

Figure 5. Effect of the addition of nicotianamine (NA) on Δ*PA4834* mutant growth in AMS. PAO1 and the Δ*PA4834* mutant were grown in AMS (white bars) and AMS amended with 100 μM NA (gray bars) and growth indices were measured. Experimental conditions were identical to those described in Fig. 1A. Five independent experiments were performed, and mean values ± SD (error bars) are displayed in each bar. *$p < 0.05$ vs. growth of the Δ*PA4834* mutant in AMS with no NA supplementation.

6. *In vivo* virulence of the Δ*PA4834* mutant is attenuated

We next examined whether *in vivo* infectivity of PAO1 was affected by deletion of the *PA4834* gene by employing the burn wound infection and airway infection mouse models. In both cases, infectivity of the Δ*PA4834* mutant was significantly compromised. The average number of viable cells recovered from the infected burn wound areas indicated that the mutant was less capable of proliferating than its parental strain PAO1 (Fig. 6A). Furthermore, while severe inflammation was clearly induced by infection with PAO1, the degree of inflammation was considerably decreased by infection with the same dose of the Δ*PA4834* mutant (Fig. 6B). The attenuation of bacterial virulence by the *PA4834* gene deletion was even more dramatic in the acute mouse lung infection model. The average number of viable cells enumerated from the infected lung of PAO1-infected mice was ~1.8×10^{8}/ml, while it was decreased to ~1.4×10^{6}/ml in mice initially infected with the same number of mutant cells (Fig. 6C), clearly suggesting that the mutant was not competent in establishing airway infection. As expected, a high degree of inflammation was only induced in the lung tissues of mice infected with PAO1, but not with the Δ*PA4834* mutant (Fig. 6D).

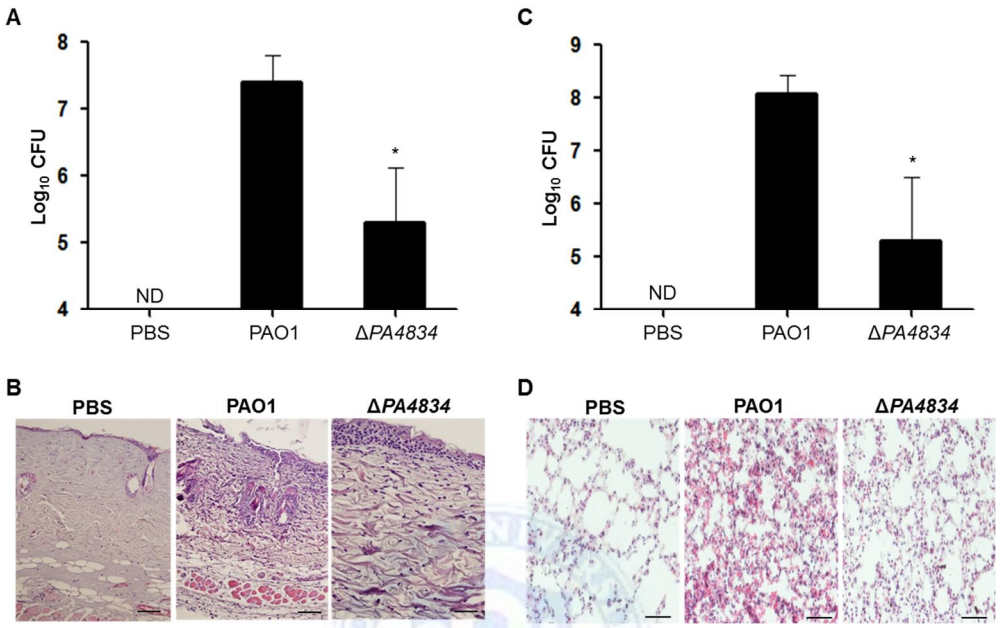

Figure 6. *In vivo* infectivity of the Δ*PA4834* mutant is decreased. (A, B) Mouse burn wound infection. (A) PAO1 and the Δ*PA4834* mutant were infected in the thermally-injured region of the mouse skin. The infection dose was 2×10^8 bacterial cells. After 24 hrs, the infected area was incised and homogenized to enumerate viable cells. Four independent experiments were performed, and mean values ± SD (error bars) are displayed in each bar. *$p < 0.05$ vs. CFU of PAO1. ND, not detected. (**B**) Histological images of infected skin sections of mice challenged with PBS (negative control), PAO1, or the Δ*PA4834* mutant. (C, D) Mouse airway infection. (C) PAO1 and the Δ*PA4834* mutant (1×10^7 CFU) were exposed to the mouse airway via the intranasal route. After 24 hrs, mouse lungs were removed and homogenized to enumerate viable cells. Four independent experiments were performed, and mean values ± SD (error bars) are displayed in each bar. *$p < 0.05$ vs. CFU of PAO1. ND, not detected. (D) Representative histological images of lung sections of mice challenged with PBS (negative control), PAO1, or the Δ*PA4834* mutant.

Ⅳ. DISCUSSION

Airway mucus is a key component of the innate immunity that mediates the elimination of inhaled microorganisms via mucociliary clearance.[33,43,44] It contains antimicrobial components such as lysozymes and defensing.[45] The bactericidal activity, however, is compromised in the diseased CF airway due to the abnormally altered mucus environment and *P. aeruginosa* is capable of establishing recalcitrant infection under such conditions.[4,9,46] Given the fact that the CF patient airway is equally exposed to various opportunistic pathogens, it remains a question as to why *P. aeruginosa* has been so successful in colonizing and proliferating in the CF patient airway.[11,47,48] Here, we addressed this long-standing question using *ex vivo* AMS experimental conditions to study the host-microbe interaction, mimicking the early stages of airway infection. *P. aeruginosa* was capable of propagating upon interaction with AMS, a finding not observed in other tested bacterial species. These data strongly suggest that *P. aeruginosa* infection in patients with CF, COPD, or ventilator care stems from the specific response of *P. aeruginosa* to the airway environment and not from the abnormal composition of the airway mucus *per se*. This notion was further verified by our findings that *P. aeruginosa* was equally capable of propagating in secretions of cultured epithelial cells derived from a CF patient (CuFi-1) or a normal individual (NuLi-1). The growth of *P. aeruginosa* in nasal secretions collected from healthy volunteers has been previously reported,[49] since then, it has been further elucidated that airway mucus provides the carbon source *P. aeruginosa* needs to proliferate.[47,50]

We then explored mechanisms by which *P. aeruginosa* is resistant to airway mucus, which is a completely different response than what is seen in other bacterial species. We focused on the PAO1 genes upregulated when exposed to AMS. Our microarray data showed that various genes related to iron uptake and metabolism were upregulated when *P. aeruginosa* is exposed to AMS. In particular, genes that

encode proteins involved in the production of siderophores such as pyoverdine and pyochelin were substantially upregulated, which is remarkably similar to previous studies where *P. aeruginosa* was exposed to airway mucus and low iron environments.[51-53] *P. aeruginosa* is known to have a special ability to utilize small amounts of iron by the synthesis of siderophores such as pyoverdine and pyochelin.[15,54-56] We also confirmed that PAO1 has higher siderophore activity than other bacteria. Based on these findings, we assumed that the selective resistance of *P. aeruginosa* to airway mucus came from the ability of *P. aeruginosa* to survive in low iron concentration conditions via a special iron-utilizing system.

To test this assumption, we screened for the critical gene(s) necessary for the survival of *P. aeruginosa* in airway mucus using transposon insertion mutants with special attention to genes regulating iron metabolism. In contrast to our expectations, transposon mutants defective in the production of pyoverdine and pyochelin, chemicals known to be important for iron acquisition, were able to survive upon treatment with AMS, while the *PA4834* mutant lost its resistance to airway mucus. The *PA4834* gene encodes a membrane-associated protein (284 amino acids) that contains a duplication of the evolutionarily conserved EamA domain, a well-characterized motif present in proteins belonging to the Drug and Metabolite Transporter (DMT) family. Of note, PA4834 has a 51% amino acid similarity to the S-adenosylmethionine (SAM) uptake transporter of *Paracoccus aminophilus* strain JCM 7686. In addition, SAM is a precursor of NA, a well-characterized phytosiderophore produced by plants.[57,58] The *PA4834* gene is part of a putative operon with the *PA4835*, *PA4936* and *PA4837* genes, and a BLASTN search demonstrated that this particular gene cluster is exclusively present in *P. aeruginosa* strains. The *PA4837* gene is predicted to encode a TonB-dependent siderophore receptor. The *PA4836* gene, although annotated as a hypothetical protein in the PAO1 genome database, likely encodes a protein homologous to NA synthase. Therefore, it was reasonable to

hypothesize that this operon is related to a new iron transport system and the possession of this system may account for the resistance of *P. aeruginosa* to airway mucus. Although the primary sequence of the PA4834 protein is similar to the SAM uptake transporter, the possibility of PA4834 acting as a SAM transporter is low because the concentration of intracellular SAM was comparable in both the PAO1 and Δ*PA4834* mutant (data not shown). Importantly, the ability of the Δ*PA4834* mutant to grow in AMS was recovered by the extraneous addition of NA. Thus, it is highly possible that PA4834 encodes a protein involved in NA secretion. Consistent with our finding, previous genome-wide studies also reported the potential importance of this genetic region for the survival of *P. aeruginosa* in the airway environment. First, the *PA4834-PA4837* operon has been shown to be upregulated in *P. aeruginosa* strain PA14 growing on artificial sputum medium as a sole carbon source.[47] Second, the *PA4837* gene was found to be expressed in *P. aeruginosa* clinical CF isolates early in CF infection.[59] Third, expression of genes in this operon was also markedly increased when *P. aeruginosa* strain PA14 was grown in the peritoneal cavity of a rat.[60] Lastly, the *PA4935* and *PA4836* genes were exclusively expressed under in *in vivo* burn wound infections.[61] These findings, together with our results, strongly suggest that the *PA4834-PA4837* operon plays an essential role in bacterial proliferation in airway mucus. Under iron-replete conditions like LB media, both PAO1 and the Δ*PA4834* mutant may sequester iron with siderophores such as pyochelin and pyoverdine. However, our results demonstrate that bacterial growth of the PAO1 mutant defective in production of these two molecules alone was not affected during incubation with AMS, while the *PA4834* gene mutation resulted in a significant defect in bacterial ability to cope with AMS-induced stress.

Although this study identified a new genetic element that accounts for the exceptional ability of *P. aeruginosa* to propagate in human airway mucus, our study does have the following limitations. First, because AMS samples were only provided

in small quantities, we were only able to grow *P. aeruginosa* strains for a short period of time. Considering that *P. aeruginosa* establishes chronic biofilm infection in the CF airway,[44] our experiments using AMS may not be completely relevant to describe *P. aeruginosa* pathogenesis *in vivo*. Second, because AMS samples were prepared from outpatients with varying degrees of health conditions, differential antimicrobial activities were observed among the AMS preparations. Regardless, PAO1 cells were always resistant to AMS treatment while the Δ*PA4834* mutant exhibited susceptibility to the same treatment. These difficulties could be overcome by constructing a stable airway cell line that secretes mucus with predictable composition. Lastly, deletion of the *PA4834* gene resulted in a dramatic phenotype in response to AMS incubation and the sole addition of NA completely rescued the ability of the Δ*PA4834* mutant to grow in AMS. However, more experiments are necessary to precisely understand the molecular basis of how NA is produced, secreted, and internalized to act as a siderophore and how each gene product of the operon contributes to this process.

Ⅴ. CONCLUSION

In conclusion, our data show that *P. aeruginosa* can selectively grow in airway mucus by utilizing a newly identified iron uptake system. Our present results provide fundamental insight into the competent behavior of *P. aeruginosa* under airway-specific environments. Selective growth of *P. aeruginosa* in the CF airway is caused by the ability of this important pathogen to manage adverse mucus conditions, such as low iron availability, and not due to the compositional changes of the airway mucus *per se*. Therefore, any condition where the mucociliary clearance system is impaired in patients with CF, COPD, or prolonged ventilator use can lead to *P. aeruginosa* infection. Our findings also suggest that the suppression of *PA4834* gene expression or blocking the biological activity of the *PA4834* gene product can be novel approaches to the treatment of *P. aeruginosa* infections.

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ABSTRACT (IN KOREAN)

기도점액에서 녹농균이 생장하는데 필수적인 역할을 하는 새로운 철분포획체 시스템

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기 미 아

녹농균은 낭포성 섬유증 환자에서 호흡기 감염을 일으키는 균으로 치명적이다. 본 논문에서는 초대 배양된 정상 사람의 기도 상피 세포로부터 분비되는 기도점액 분비물과 녹농균의 상호작용에 대하여 연구하기 위하여 기도점액 분비물에서 여러 균들을 배양해 보았다. 실험 결과 균들은 기도점액 분비물에서 살아남지 못했다. 하지만 녹농균의 원형인 PAO1은 기도점액 분비물에서 생존 할 뿐만 아니라 증식하였다. 이러한 결과에 영향을 미치는 유전적 요소를 찾고자 마이크로어레이 기법을 이용하여 기도점액 분비물에 반응하여 증가하는 PAO1의 유전자를 분석하였다. 증가된 유전자가 각각 돌연변이 된 PAO1을 기도점액 분비물에서 배양하였다. 그 결과 PAO1의 *PA4834* 유전자가 조작된 Δ*PA4834* 돌연변이체가 기도점액 분비물에서 다른 균들처럼 생존하지 못하였다. 이 Δ*PA4834* 돌연변이체는 100 μM의 고농도의 제 2철을 첨가하였을 때 재생장 하였다. 또한 기도점액 분비물에서 배양한 후 세포 내 철의 양을 측정해 보았을 때 PAO1과 비교하여 Δ*PA4834* 돌연변이체의 세포 내 철의

양이 현저히 감소되어 있었다. 이러한 결과들은 *PA4834* 유전자가 철 획득을 위해 중요한 역할을 할 것이라는 것을 보여준다. 녹농균은 기능이 잘 알려진 철분포획체인 pyoverdine과 pyochelin을 철 획득을 위하여 주로 이용한다. 하지만 이 두 철분포획체를 분비하지 못하도록 조작된 PAO1의 돌연변이체는 기도점액 분비물에서 생장하였다. *PA4834* 유전자와 같이 조절되는 오페론의 유전자 중 *PA4836* 유전자는 식물의 철분포획체인 nicotianamine을 합성하는데 관여할 것으로 여겨지고 있다. 본 연구에서, nicotianamine (100 μM)을 기도점액 분비물에 첨가하였을 때 Δ*PA4834* 돌연변이체가 재생장 하는 것을 확인하였다. 이러한 결과들은 *PA4834* 유전자가 새로운 철분포획체 시스템의 일원으로 기도점액 분비물에서 PAO1이 선택적으로 증식하는데 있어 중요한 요소임을 보여준다. 또한 Δ*PA4834* 돌연변이체에 의한 *in vivo* 감염성은 PAO1과 비교하여 감소한다는 것을 확인하였다. 종합하면, 본 논문은 기존에 밝혀지지 않은 새로운 철 획득 시스템을 코딩하는 녹농균의 유전인자를 밝혔고 이는 호흡기 감염에 일차적인 방어체계인 기도점액에서 녹농균이 생존하는데 중요한 요소임을 보여주고 있다.

핵심되는 말: *PA4834* 유전자, 철분포획체, 기도점액 분비물

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